Study of surface interactions between peptides, materials and bacteria for setting up antimicrobial surfaces and active food packaging

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Abstract
Active food packaging and antimicrobial surfaces are innovative solutions for increasing food-borne diseases and changes in food habits. This review describes the different methods for setting up active packaging, with specific focus on antimicrobial surfaces developed by peptide adsorption. The key elements in the study of peptide adsorption on surfaces are the peptide nature, the surface properties and the interactions between them. Nisin, a well-known peptide, was reviewed and can be considered as a model for peptide studies. Plasma surface treatments were evaluated as potential versatile tools to provide specific functional groups and various surface characteristics needed to optimize and understand the adsorption behavior. The surface characterization methods were screened and discussed in terms of their relevance to investigate the interactions between peptides and surfaces, confirm each modification step and evaluate the antimicrobial activity of surfaces. For each factor, the advantages, drawbacks, applications, and further considerations were assessed in the fields of interaction studies and active food packaging technologies.

Keywords: Active food packaging; nisin; plasma treatment; peptides adsorption; surface interactions; surface characterization techniques.

1. Introduction
Food-borne illnesses and microbial contamination are still issues of major worldwide concern despite the introduction of modern technologies and safety concepts in the food industry. Novel strategies for food preservation include setting up active packaging and antimicrobial surfaces. Active packaging can be defined as a mode of packaging in which the package, the product and the environment interact to prolong the shelf-life or enhance the safety or sensory properties of the product, while maintaining its quality [1]. Among the several types of available active packaging, the antimicrobial version is of great importance [2].

Various kinds of active substances can now be added to the packaging material to improve its functionality and give it new or additional function. Setting up antimicrobial surfaces by peptide adsorption presents many advantages. It permits to prevent surface contamination by killing the bacteria attempting to attach on surfaces. It is also of particular interest for basic studies of peptides-materials-bacteria interactions, which provide an essential basis for the development of other more sophisticated antimicrobial systems. Besides its potential for antimicrobial food packaging applications, it can be used to cover surfaces of food processing equipment so that they self-sanitize during use [3]. This method has in addition found applications in the biomedical sector to prevent microbial growth on medical and implanted devices [4-6]. The study of peptides adsorption on surfaces requires mainly the following: (1) choice of peptide and peptides characterization, (2) surface modification, and (3) surface characterization.
Of all the antimicrobial peptides known, nisin is presently the only one commercially available and approved by the FDA (Food and drug administration) and WHO (World health organization) [7]. Nisin is a bacteriocin, naturally produced by Lactococcus lactis subsp. Lactis. This peptide is effective against a wide range of Gram-positive bacteria [8]. It has also shown the ability to retain its antibacterial activity in the adsorbed state [9]. Other bacteriocins have been isolated and can act as effectively as nisin with respect to particular foods/target bacteria. However, they have not been studied and exploited to the same extent as nisin. Therefore, nisin can serve as a model or case study encouraging the emergence of new bacteriocins and new potential bio-preservatives.

Peptides adsorption behavior is largely controlled by surface characteristics. This means that surface modification plays a vital role in the effectiveness of activated materials. Surface modification can be achieved by chemical and physical methods. Wet chemical methods using strong acids and bases have been used industrially, but the disposal of hazardous waste leads to environmental and safety problems [7,10]. As a result, physical surface modification methods are preferred and include: flame, corona, irradiations, ultraviolet (UV), plasma, and laser treatments. Plasma treatment is probably the most versatile surface treatment technique. Different types of plasma can provide a wide range of surface modifications and can be used to create new specific surface functionalities, morphologies and chemistries that will result in different surface interactions [11,12]. This is highly valuable to study and enhance peptide adsorption on surfaces. They can in addition improve the wettability, sealability, adhesion, barrier, and many other characteristics of food packaging materials, while maintaining desirable bulk properties of the polymer [11].

After functionalization, characterization of the surface-engineered materials has profound scientific importance, leading to understanding the interactions taking place between peptides, materials, and bacteria. Examples of methods used for such purposes involve: X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) to reveal the surface chemical structure of materials, atomic force microscopy (AFM) to investigate the topography of surfaces, colorimetric assays to quantify the amounts of surface functional groups and adsorbed peptides, as well as antimicrobial tests to assess the surfaces’ antimicrobial activity.

In the first part of this paper, the various kinds of active packaging and the different classes of antimicrobials that can be used for antimicrobial packaging are briefly presented. The second part focuses on peptides adsorption on surfaces. Nisin’s characteristics, mode of action, antibacterial activity, resistance, applications, as well as its specific adsorption on surfaces are discussed. Then, the general principles, advantages and disadvantages of different plasma processes that can find potential applications in food packaging and interactions studies are assessed. The following section describes the most widely used surface characterization methods and highlights their advantages, limitations and applications with respect to adsorption studies. Finally, general further considerations in this field are addressed.

2. Different methods for setting up active food packaging
Active packaging concepts provide additional specific functions in food preservation as compared to traditional passive packaging limited to hold the food product and protect it from the external conditions [13]. A proposed classification of the different active packaging systems is shown in figure 1 and each type is briefly described below. Even though the active packaging systems are not all antimicrobials, they can contribute indirectly to food preservation and inhibition of bacterial growth.

2.1. Addition of sachets, pads or tablets containing the active substances into the packaging
The atmosphere within packaging can be changed by incorporating active substances into the package using a sachet, pad or tablet and allowing mechanisms like evaporation and absorption processes to inhibit the microbial growth. Such sachets are enclosed loose or attached to the interior of a package and their common disadvantages are the risks of sachet leakage and that the sachets can be accidentally ingested [7]. The most commercial applications include oxygen scavengers, carbon dioxide scavengers and generators, moisture absorbers, ethanol and chlorine dioxide generators.

2.1.1. Oxygen scavengers
Oxygen scavengers are primarily used to prevent oxidation, microbial growth, and spoilage reactions in foods [14]. Although they may not be intended to be antimicrobial, a reduction in oxygen inhibits the growth of aerobic bacteria and molds [3]. Oxygen scavenging technology may be used to remove residual O2 after modified atmosphere packaging (MAP) or vacuum packaging and to absorb the oxygen that permeates through
the packaging film [2]. However, under certain circumstances, their use can promote the growth of facultative or anaerobic microorganisms, which present another big threat to food safety [7]. Such types of packaging are relevant for meat, bakery, pasta, dairy, and produce industries [1,3,14].

2.1.2. Carbon dioxide scavengers and generators
Carbon dioxide generators are considered as antimicrobials too because of their inhibitory activity against a range of aerobic bacteria and fungi [2]. However, it has been reported that a high concentration of carbon dioxide decreased the growth rate of Clostridium botulinum but greatly increased its production of toxin [15]. Thus, research into the safety risks associated with the use of carbon dioxide in packaging systems is necessary. Most applications for this packaging are for meat and poultry preservation [2]. Carbon dioxide absorbers may be used to remove carbon dioxide during storage in order to prevent bursting of the package. Possible applications include their use in packs of dehydrated poultry products and beef jerky [14].

2.1.3. Moisture absorbers
Moisture absorbers are used to prevent water condensation and reduce the water activity of the product, thereby indirectly affecting microbial growth. They have been successfully used for moisture control in a wide range of foods, such as cheeses, meats, chips, nuts, popcorn, candies, gums, and spices [1,3].

2.1.4. Ethanol generators
Ethanol is used routinely in medical and pharmaceutical packaging applications, indicating its potential as an antimicrobial. Ethanol generators retard molds and prevent microbial spoilage of intermediate moisture foods as cheese, bakery, and dried fish products [1,3].

2.1.5. Chlorine dioxide generators
Chlorine dioxide has an antimicrobial activity against a broad spectrum of microorganisms including bacteria, spores, fungi, and viruses. Sustained and controlled release of chlorine dioxide is related to exposure to humidity greater than 80% and light. Applications for this technology are just beginning to unfold in the food industry for meat, poultry, fish, dairy, confectionery, and baked goods [2]. However it has an adverse effect on meat quality including color darkening [7].

2.2. Direct incorporation of the antimicrobial agents into the packaging material
Many antimicrobials can be directly incorporated into the packaging material, particularly films. Thermally stable antimicrobials as silver substituted zeolites and triclosan can be added in the melt for extrusion, co-extrusion or injection molding during the polymer/film processing [16,17]. For heat-sensitive antimicrobials like enzymes, solvent compounding may be a more suitable method for their incorporation into polymers [3]. However, all those packaging materials must be in contact with the food and a migration of the antimicrobial agents to the surface is expected [2]. For volatile antimicrobials as sulfur dioxide and allyl isothiocyanate, precursor molecules are incorporated directly into the polymer or into carriers that may be extruded into packaging materials. The theoretical advantage of volatile compounds is that they can penetrate the bulk matrix of the food and that the contact between the food and the packaging is not necessary [3].

Antimicrobials can be incorporated into multilayers films (control layer/matrix layer/barrier layer) to achieve appropriate controlled release to the food surface. The inner layer controls the rate of diffusion of the active substance, while the matrix layer contains the active substance and the barrier layer prevents migration of the agent towards the outside of the package [3,18]. The main drawback of this method is that the embedded antimicrobials in the matrix layer will lack direct contact with the surrounding bulk, and will need to be efficiently released through diffusion to the interface. The diffusion process of the agents in the multilayer architecture is more complex than diffusion in solutions. Additional factors such as the tortuosity of the diffusion pathway, assembly thickness and peptide–polymer interactions can significantly impact the diffusion process. Furthermore, binding of bacteria on the top assembly layer may block the exit of bioactive molecules, which are still entrapped within the matrix [18].

Recent developments in nanotechnology allow also the incorporation of bioactive nano-compounds into the film for food packaging applications [7,19]. However the use of nano-composites has been a concern owing to the potential hazard of inhaled or ingested nano-materials and to the insufficient database and information on their toxicity [7].
2.3. Coating of the antimicrobial agents on the surface of the packaging material
An alternative to the incorporation of antimicrobial compounds during extrusion is to apply the antimicrobial additives as a coating. This has the advantage of placing the specific antimicrobial additive in a controlled manner without subjecting it to high temperature or shearing forces [2,3,14]. In addition, the coating can be applied at a later step, minimizing the exposure of the product to contamination. The coating can serve as a carrier for antimicrobial compounds in order to maintain high concentrations of preservatives on the surface of foods. Bioactive agents’ activity may be based on migration or release by evaporation in the headspace. Further research is required to establish the parameters for optimal antimicrobial efficiency, adhesion on packaging support, or the desorption procedure from the materials. Such factors as levels of antimicrobial agents, biocide purity, plastic formulation, and varying plastic composition will need to be evaluated [2].

2.4. Natural or modified antimicrobial polymers
Some polymers are inherently antimicrobials while others need to be modified to render them antimicrobials.

2.4.1. Natural antimicrobial polymers
Inherently antimicrobial polymers with film-forming properties provide bioactive films and can at the same time, be used as carriers of other antimicrobials. Cationic polymers such as chitosan and poly-L-lysine exhibit antibacterial activity since charged amines interact with negative charges on the cell membrane, causing leakage of intracellular constituents [2,3].

2.4.2. Modified antimicrobial polymers
In contrast to naturally antimicrobial polymers, some bioactive materials have been produced by modifying the surface composition of the polymer. A conversion of amide to amine groups of nylon by electron irradiation achieved an antimicrobial activity that inactivated target cells by contact [20]. Plasma treatments are under development as well. Ozdemir and co-workers [21] indicated that fluorine-based plasmas may be used to fluorinate the surface of polymers and form a packaging with "self-sterilization" or "self-pasteurization" capabilities. However, further research needs to be done to establish the effectiveness of such treatment.

2.5. Bioactive edible films and coatings
There is a growing interest in edible coatings due to factors such as environmental concerns, need for new storage techniques, and opportunities for creating new markets for under-utilized agricultural commodities with film-forming properties. Edible films and coatings prepared from polysaccharides, proteins, and lipids have a variety of advantages such as biodegradability, edibility, biocompatibility, aesthetic appearance, and barrier properties against oxygen and physical stress [14,17]. For active packaging applications, the incorporated active agents are limited to edible compounds. The antimicrobials should be safe and approved as a food additive, because they have to be consumed with the coating layers and foods together. Cellulose derivatives such as hydroxyl-propyl-methyl-cellulose (HPMC) and alginate coatings are promising raw materials for edible coatings associated with antimicrobial entities [2,17]. The potential applications of edible films include preservation of fresh, frozen and processed meat and poultry products [2,7,17].

2.6. Attachment of the antimicrobials agents on the surface of the packaging material
Bioactive molecules can be attached onto polymers either chemically (via covalent immobilization) or physically (via adsorption) for setting up active antimicrobial surfaces. In the first category, the antimicrobial agent does not migrate to the surface of the food. In the second one, the antimicrobial effect is achieved with migration. Moreover such antimicrobial surfaces are intended not only for food applications but also for biomedical applications [22].

2.6.1. Antimicrobials covalent immobilization on surfaces
Chemical methods of immobilization involve the formation of at least one covalent bond between the bioactive molecules and the polymer matrix. This requires the presence of functional groups on both the antimicrobial and the polymer [3]. The antimicrobials with functional groups and with molecular structure large enough to retain activity in such applications are limited to enzymes or other antimicrobial proteins [17]. The most commercial polymers are inert with no reactive groups and they must thus undergo surface functionalization prior to attachment of a bioactive compound. Surface treatments using plasma techniques can be used for this purpose.
and will be discussed later. In addition, immobilization usually requires the use of cross-linkers or “spacer” molecules that link the functionalized polymer surface to the bioactive agent [3,22]. The advantages of this method are creating a stable bond between the compound and the functionalized polymer surface and providing activity during a sustained period of time. For active food packaging applications, it ensures that the bioactive compound will not migrate to the food and thus may offer the regulatory advantage of not requiring approval as a food additive but it still need to be accepted for direct contact with the food [2,22]. However, a consistent problem with the immobilized bioactive compounds is the decrease in their activity [18,22-24]. Covalent binding may alter the conformational structure and the active centers of the bioactive molecules as peptides and enzymes [24]. It may also restrict the mobility of peptides affecting thus their mode of action normally involving insertion and disruption of the cell membrane of the target micro-organism [25]. This reduced activity can significantly compromise the effectiveness of activated surfaces and shows the need for detailed fundamental studies to understand and optimize the parameters affecting the antimicrobial performance of immobilized bioactive agents. Such parameters include surface concentration of bound antimicrobials, spacer length, spacer flexibility, spacer cleavage due to polymer degradation reactions, and effect of peptide orientation at the interface on its biological activity [18].

2.6.2. Antimicrobials adsorption on surfaces

Physical methods of adsorption are mainly governed by hydrogen bonding, van der waals forces, electrostatic and hydrophobic interactions between the antimicrobials and the surfaces. Peptides are the most well-known antimicrobials with ability to adsorb on surfaces [25]. The factors affecting peptide adsorption on surfaces are numerous and entail: the peptide characteristics (type, charge, size, conformational stability…), the surface properties (nature, composition, charge, topography, roughness, hydrophobic/hydrophilic character…), the surrounding medium (pH, ionic strength), and the interactions between them. Moreover, peptide adsorption on surfaces can be optimized by the proper combination of treatment conditions such as contact time, peptide concentration, pH of the solution, and adsorption temperature [25-28]. However, such non-covalent methods are effective for short-term applications, because of the limited availability and relatively quick release of antimicrobials from the polymer. This method is the main focus of this review and will be further detailed for specific nisin (peptide) adsorption on surfaces.

![Classification of the different active packaging systems.

Figure 1: Classification of the different active packaging systems.

3. Classes of antimicrobials

Antimicrobial packaging is a form of active packaging that requires the presence of antimicrobials in order to be effective. The different antimicrobial agents that can be used in antimicrobial films, containers, and utensils are presented in previous reviews [1-3,7,17] and are listed in table 1.
Table 1: Examples of antimicrobial agents for potential use in food packaging materials

<table>
<thead>
<tr>
<th>Antimicrobial agents classes</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic acids</td>
<td>Acetic acid, benzoic acid, p-aminobenzoic acid, citric acid, lactic acid, malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid</td>
</tr>
<tr>
<td>Organic acid salts</td>
<td>Potassium sorbate, sodium benzoate, potassium lactate</td>
</tr>
<tr>
<td>Organic acid anhydrides</td>
<td>Benzoic anhydride, sorbic anhydride</td>
</tr>
<tr>
<td>Inorganic acids</td>
<td>Phosphoric acid</td>
</tr>
<tr>
<td>Inorganic gases</td>
<td>Sulfur dioxide, chlorine dioxide</td>
</tr>
<tr>
<td>Alcohols</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Amines</td>
<td>Hexamethylenetetramine (HMT)</td>
</tr>
<tr>
<td>Ammonium compounds</td>
<td>Silicon quaternary ammonium salt</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Natamycin</td>
</tr>
<tr>
<td>Antimicrobial peptides</td>
<td>Defensin, magainin, attractin, cecropin</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Butylatedhydroxyanisole (BHA)</td>
</tr>
<tr>
<td>Bacteriocins</td>
<td>Bavaricin, brevicin, carnocin, lacticin, mesenterocin, nisin, pediocin, sakacin, subtilin</td>
</tr>
<tr>
<td>Chelators</td>
<td>Citrate, conalbumin, ethylenediaminetetraacetate (EDTA), lactoferrin, polyphosphate</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Chitinase, ethanol oxidase, β-glucanase, glucose oxidase, lysozyme, myeloperoxidase</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Lauric acid, palmitoleic acid, glycerol mono-laurate</td>
</tr>
<tr>
<td>Fatty acid ester</td>
<td>Monolaurin (lauricidin⁶)</td>
</tr>
<tr>
<td>Fungicides</td>
<td>Benomyl, imazalil</td>
</tr>
<tr>
<td>Metals</td>
<td>Copper, silver, zirconium, titanium oxide</td>
</tr>
<tr>
<td>Plant and spices extracts</td>
<td>Allyl isothiocyanate (AITC), grapefruit seed extract, bamboo powder, rheum palatum, coptischinensis extracts, cinnamic acid, caffeic acid, p-coumaic acid</td>
</tr>
<tr>
<td>Essential oils and plant-volatile components</td>
<td>Carvacrol, cineole, cinnamaldehyde, citral, p-cymene, estragole (methyl chavicol), geraniol, Hinokitiol (β-thujaplicin), linalool, terpineol, thymol, oregano, lemongrass</td>
</tr>
<tr>
<td>Natural phenols</td>
<td>Catechin, p-cresol, hydroquinones</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Butylatedhydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ)</td>
</tr>
<tr>
<td>Parabens</td>
<td>Ethyl paraben, methyl paraben, propyl paraben</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Chitosan, konjac glucomannan</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Chitooligosaccharide</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Reuterin, triclosan, nitrites and sulphites, probiotics</td>
</tr>
</tbody>
</table>

4. Nisin

Among the different classes of antimicrobials, bacteriocins represent an attractive choice with regard to consumers demanding “natural and healthy products” and avoiding “artificial and chemical preservatives”. Bacteriocins are antibacterial peptides produced by bacteria and can kill or inhibit the growth of other bacteria (usually closely related species) [29]. Many lactic acid bacteria produce a high diversity of different bacteriocins [30]. Although several bacteriocins have been characterized, nisin remains the most studied and the most commercially important bacteriocin because it is the only one approved for food applications and it has gained widespread application in the food industry. Nisin has been added to the GRAS (Generally recognized as safe) list by the United States FDA (Food and drug administration) and to the positive list of food additives by the EU (European Union) where it was assigned the number E234. It has also been accepted by the FAO (Food and agriculture organization) and the WHO (World health organization). The peptide has been used as a food preservative in almost 50 countries for over 40 years because of its non-toxicity, high antibacterial activity,
immediate digestibility in the intestine by enzyme α-chymotrypsin, heat stability at low pH, and absence of color and flavor [31,32].

4.1. Nisin characterization
Nisin is a ribosomally synthesized and post-translationally modified lantibiotic, produced by Lactococcus lactis subsp. Lactis [33]. Lantibiotics are class I bacteriocins characterized by intramolecular rings formed by the unusual thioether amino acids lanthionine and 3-methyl-lanthionine, and also contain other rare dehydrated amino acids including dehydroalanine (Dha) and dehydrobutyrine (Dhb) [34,35]. Post-translational modification renders the lantibiotics biologically active [32]. In addition, nisin is a 3.5 kDa cationic amphiphilic peptide with a net positive charge. It contains 34 amino acids distributed in clusters of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus end [36] (Fig. 2). Nisin A is the originally isolated form of nisin and a further five natural variants have been described and differ by up to 10 amino acids (of 34 in total in nisin A). Nisin Z, F, and Q are like nisin A produced by Lactococcus lactis, while nisin U and U2 are produced by Streptococcus sp. [37].

Figure 2: The primary structure of nisin A showing the distribution of amino acids in the hydrophilic and hydrophobic sides of the molecule (adapted from [38]).

4.2. Nisin mode of action
The antibacterial activity of bacteriocins is based on interaction with the targeted cell membrane of sensitive mainly Gram-positive bacteria. The prototype lantibiotic nisin is active at nanomolar concentrations through different killing mechanisms that are combined in one molecule. It inhibits cell wall biosynthesis and forms pores in the membrane through specific interactions with the cell wall precursor lipid II. The general steps involved in nisin activities include i) binding to the bacterial membrane, followed by ii) insertion into membrane, iii) pore formation, and iv) interactions with lipid II. We will describe briefly the steps responsible for nisin potential activity.

i) Nisin binding to the bacterial membrane
Binding with the target membrane is the first step in lantibiotic’s mode of action. The positively charged C-terminus of nisin binds via electrostatic interactions with the anionic lipids of the bacterial cell membrane [29,32,39,40].

ii) Nisin insertion into membrane
After binding to the membrane, the hydrophobic interactions allow the amphiphilic peptide to insert its hydrophobic N-terminus into the lipid phase of the membrane, while the peptide adopts an overall orientation parallel to the membrane surface [29,39]. The most hydrophobic N-terminus of the peptide mainly affects nisin insertion and the whole peptide antimicrobial activity [40]. The presence and the increase of concentration of anionic lipids are essential for respectively efficient and deeper insertion of nisin in the lipid phase of the membrane [39,40].

iii) Pore formation by nisin
The inserted nisin subsequently obtains a trans-membrane orientation without losing contact with the membrane surface and thereby distorts the lipid bilayer to form a short-lived pore. The pore formation results in the rapid efflux of cellular materials (e.g. ions, amino acids, ATP), leading to the cell death [41]. The formed pores are of transient nature and the nisin-induced leakage is paralleled by translocation of the whole nisin molecule to the
inside of the membrane [30,39]. It is also assumed that insertion is followed by aggregation and association of several molecules with the membrane to form a pore since lantibiotics are small peptides [40].

iv) Nisin interactions with lipid II
In addition to pore formation mechanism, a factor present in the target membrane dramatically increases the nisin activity and explains its effectiveness at nanomolar concentrations. It was found that nisin uses lipid II, a peptidoglycan precursor, as a docking molecule for pore formation and binds with it as well for inhibiting cell wall biosynthesis [39,40]. Several experiments suggested that the N-terminus and the ring structures of nisin are important for its specific interaction with Lipid II [39].

Moreover, lipid II is the high affinity target for nisin. The dissimilar sensitivities of different indicator strains to nisin and the different minimum inhibitory concentration (MIC) values may be due to the presence of different lipid II contents among various microorganisms [29,32,39].

Lantibiotics are also effective against spores; using another distinct activity mechanism with a different structure-function relationship. The target of nisin for the inhibition of spore outgrowth is provided by reactive thiol groups on the spores [32]. Nisin activity against spores is attributed to the dehydroalanine residue in position 5 of the peptide and to binding with sulfhydryl groups on the exterior of the spores [31,32,40]. It is the combination of these properties that make nisin such a unique effective molecule.

4.3. Nisin antimicrobial activity
Nisin has shown to be effective against a wide range of Gram-positive bacteria, including many important foodborne pathogens such as Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, and Clostridium botulinum [8,30,42]. In addition, it inhibits the outgrowth of spores from several Bacillus and Clostridium species [31]. However, it shows little or no activity against Gram-negative bacteria [43]. The described nisin mode of action showed that the cytoplasmic membrane is the target for nisin in vegetative cells and can thus explain such difference in activity. Unlike Gram-positive bacteria, Gram-negative species are covered and protected by the presence of an outer membrane carpeted by the lipopolysaccharide layer that acts as a barrier to the action of nisin on the cytoplasmatic wall [31]. Moreover, Gram-positive bacteria have relative higher concentrations of anionic lipid in their cytoplasmic membrane, facilitating nisin insertion, as compared to Gram-negative species [39].

Consequently, there is considerable interest in using nisin in combination with other antimicrobials and treatments to expand its spectrum of activity. For example, Gram-negative bacteria can be sensitized to nisin by exposure to chelating agents, sub-lethal heat, and to freezing [43]. When the outer membrane is impaired by agents such as the food-grade chelator EDTA, it is disrupted rendering Gram-negatives sensitive to bacteriocins [30].

4.4. Resistance mechanisms
Today, bacteriocins are largely considered as a potential answer to the growing problem of resistance to conventional antibiotics [32,44]. However, when a new preservative is found to be safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. It is therefore important to understand the mechanism of resistance so that it can be avoided. Antibiotic resistance is usually associated with a genetic determinant, facilitating the transfer of resistance between cells, strains, and species. In contrast, bacteriocin resistance results mostly from a physiological adaptation and a change in the target cell membrane composition [45,46]. Moreover, several bacterial species can produce an enzyme, nisinase, which degrades nisin [47]. Consequently, the abuse of bacteriocins in food may induce cells adaptation and resistance and their usage should be optimized rather than maximized.

4.5. Nisin applications
  4.5.1. Food applications
Nisin has found applications as a preservative and shelf-life extender in a broad range of food products which include processed and cottage cheese, milk products, dairy desserts, liquid egg, canned vegetables, salad dressings, high moisture hot baked flour products, fish and meat products, confectionary, beer and wine manufacture [41,43,44].

However various factors in food can affect or partially affect the action of nisin. For example nisin interactions with the food matrix and high fat content in foods can reduce its activity [2,43]. One of the advanced ways for using the bacteriocin is setting up nisin-activated antimicrobial packaging. Moreover, such systems can reduce the negative interactions or dilution of antimicrobial compounds induced by directly dispersing or mixing them
with food. They will allow thus the peptide to be effective and maintained at higher concentrations on the food surface – where the microbial growth is mostly found – rather than lost in the food matrix [2,7].

Few examples of such successful nisin applications were presented according to the method used for preparing the antimicrobial food packaging:

- Nisin adsorption on surface [26,27].
- Nisin-containing edible films [48,49].
- Direct nisin incorporation into plastic or multilayer films [50,51].
- Nisin covalent immobilization on surface [52].
- Nisin coating on the surface of the packaging materials [53-55].

Nisin was also used as part of a multi-preservation system known to food microbiologists as hurdle technology [56]. Other factors such as low temperature, pH, additives, and preservation techniques will be combined to ensure efficient destruction or inhibition of bacteria in foods. Antimicrobial packaging can be considered then as a “final-hurdle” in a food system where other hurdles already exist [30,57]. For example, nisin-activated films combined with modified atmosphere packaging and refrigeration temperatures permitted to reduce the population of lactic acid bacteria and to extend the shelf-life of sliced cheese and ham [58]. Siragusa and co-workers [50] showed that nisin impregnated packaging held at 4°C allowed to reduce the population of the psychrotrophic bacterium *Brochothrix thermosphacta* and to control the spoilage of beef carcass. The number of spoilage populations on beef cuts was also reduced by coupling storage at 1°C with antimicrobial packaging activated by nisin, HCl, and EDTA [59].

4.5.2 Other applications

Nisin has also found applications in agricultural, personal care products, clinical and veterinary therapies [31,32,60]. Some remarkable uses of nisin include: treatment of atopic dermatitis, oral decay, stomach ulcers, colon and enterococcal infections, control of respiratory tract infections caused by *Staphylococcus aureus*, inhibition of experimental vascular graft infection caused by methicillin-resistant *Staphylococcus epidermidis*, and more interestingly, nisin inhibits sperm motility, showing its potential as a contraceptive agent. It has also been used in health care products such as toothpaste and skin care products. In veterinary therapy, nisin is currently used as sanitizer against mastitis pathogens (*Staphylococcus* and *Streptococcus* species).

The success of adsorbed nisin on food contact surfaces prompted further studies to extend applications on medical devices [4]. Bower and co-workers [5] used nisin for setting up antimicrobial implantable medical devices. Nisin was adsorbed on polyvinyl chloride (PVC) suction catheter tubing and was exposed to three species of Gram-positive bacteria. Nisin-treated PVC tubing demonstrated an ability to inhibit bacterial growth, while the untreated tubes allowed attachment and growth of the pathogens. They confirmed as well the ability of nisin to retain its activity in vivo and studied its activity when applied on implants placed in sheep and ponies. Nisin was also adsorbed on silica microspheres treated with trichlorovinylsilane to introduce hydrophobic vinyl groups, followed by self-assembly of the polyethylene oxide–polypropylene oxide–polyethylene oxide (PEO–PPO–PEO) triblock. The triblock-coated silica permitted the enhancement of nisin resistance to elution by fibrinogen blood proteins [6].

Although the main nisin applications are in food as natural agent preservative, research has verified its effectiveness for therapeutic purposes too. The peptide also showed its potential use for setting up antimicrobial surfaces for both the food and medical sectors.

4.6. Study of nisin adsorption on surfaces

Nisin has shown its ability to set up antimicrobial surfaces by adsorbing on surfaces and retaining its activity. However, its adsorption on hydrophilic and hydrophobic surfaces is still a matter of debate. Proteins and peptides adsorption on surfaces remains a complicated phenomenon governed by the interactions between the peptides and surfaces. Understanding the factors controlling such interactions is essential for setting up effective antimicrobial surfaces.

Hydrophilicity/hydrophobicity may be the initial parameter affecting peptides/protein adsorption. Previous studies showed that the highest activity was observed on the most hydrophobic nisin-activated films [26]. However, these results were in disagreement with reports showing that antimicrobial activity of adsorbed nisin depended upon surface hydrophobicity, with surfaces of low hydrophobicity retaining more nisin activity than the more hydrophobic surfaces [25,61-64]. Kim and co-workers [65] also found an increase in the antibacterial activity against *Listeria monocytogenes* when nisin was adsorbed onto a hydrophilic surface. Bower and co-workers [62] reported that the low-hydrophobicity surfaces generally displayed more nisin
activity than higher-hydrophobicity surfaces, despite the finding that peptides adsorbed in greater amounts on the more hydrophobic surfaces. Similar studies on the adsorption of proteins on surfaces showed that the amount of adsorbed albumin and fibrinogen increases as the surface becomes more hydrophobic, with the adsorbed protein undergoing greater conformational changes on hydrophobic as compared to hydrophilic surfaces [66,67]. This was inconsistent with other findings showing that the amount of adsorbed nisin was higher on the hydrophilic surface than on the hydrophobic one [9,61,68].

Other factors can also influence peptides/proteins adsorption on surfaces as the electrostatic attraction and repulsion between the charged surfaces and charged proteins. In the latter case, the pH of the surrounding medium can define the charge of the peptide (pH below or above the isoelectric point) and the charge of the surface (pH below or above the pKa of functional surfaces). In general, a negatively charged surface prevents adsorption of proteins with the same sign of charge, but accelerates adsorption of proteins with the opposite sign [25]. However, when the interaction forces were measured between an acrylic acid-grafted surface and probe tips with fixed albumin or lysozyme using an atomic force microscope, a significant adhesion force was observed not only with positively charged lysozyme, but also with negatively charged albumin at physiological pH [69].

The changes in surface topography and roughness could play a role too. The surface chemistry and topography can affect proteins adsorption on surfaces [70]. The change in surface roughness can enhance the anchoring effect and the adhesion properties of a surface [71].

Therefore studying these factors and modulating the material’s surface properties such as chemical composition, hydrophilicity/hydrophobicity, surface charge and roughness, etc. are needed to preserve the antimicrobial activity of peptides and increase the activity of functionalized surfaces.

5. Surface modification by plasma treatments

Plasma treatments offer a wide range of processes that permit a tailor-made modification of surfaces for intended applications. Plasma is a partially or fully ionized gas with a net neutral charge and is often referred to as the fourth state of matter [72]. It can be divided into two main categories: Thermal plasmas (near-equilibrium plasmas) and cold plasmas (non-equilibrium plasmas). Thermal plasmas are composed of very high temperatures electrons and heavy particles, both charged and neutral, and they are close to maximal degrees of ionization (100%). Cold plasmas are characterized by low temperature heavy particles (atomic, molecular, ionic, neutral and radical species), relatively high temperature electrons, and they are associated with low degrees of ionization (10^4–10%). The system does not thus reach the equilibrium because of the difference in temperature between the particles [24]. Thermal plasmas will not be detailed in this review because they are not suitable for polymers’ applications or for the processing and surface modification of organic materials. Cold plasmas are typically carried out at low pressures and they are generated and sustained by the transfer of energy to a gas environment. This energy can be in several forms including: thermal, electric or magnetic fields and direct current, radio or microwave frequencies [73]. Radio-frequency (RF) at 13.56 MHz is one of the most widely used sources [24]. The chemical reactions in cold plasma are initiated by the “hot” highly energetic electrons. The generated ionized and excited species induce plasma-surface reactions and change the surface properties of all polymer materials [12,74]. Concerning the plasma-surface interactions, further sub-categories exist and there are different views on how they can be classified. In this paper we will group them in the three most relevant processes for food packaging and surface analysis applications. They include: plasma functionalization of polymer surfaces, plasma-induced grafting, and plasma polymerization. For convenience, when “plasma” is used alone in this paper, it refers to “cold plasma”, since thermal plasma is out of the scope of this work.

5.1. Plasma functionalization of polymer surfaces

When a polymeric material is exposed to plasma, many functional groups can be created on the surface. The active plasma species break the covalent bonds at the surface, leading to hydrogen abstraction and formation of surface radicals. The latter can then react with the gas-phase species to form different chemically active functional groups (mostly polar) at the surface [72] (Fig. 3). The type of functionalization imparted can be varied by selection of plasma gas and operating parameters.

Different reactive and inert gases are often used alone or in combination as air, argon, hydrogen, helium, oxygen, nitrogen, ammonia, carbon dioxide [21]. Oxygen and oxygen-containing plasmas are most commonly employed to produce a variety of oxygen functional groups, including C-O, C=O, O=C=O, C-O-O, and CO₂ at the polymer surfaces [10,71]. Oxygen and carbon dioxide plasmas can introduce carboxyl groups on surfaces [22]. Air and water plasmas oxidize surfaces, while water plasma can additionally incorporate hydroxyl
Plasma functionalization can be used either directly for end-use applications or as a pretreatment for subsequent surface modification techniques and attachment of bioactive agents.

**Figure 3:** Schematic illustrating the concept of the plasma functionalization process.

5.2. **Plasma-induced grafting**
Polymer surfaces can also be modified by “plasma-induced grafting”, which is a combination of plasma functionalization and conventional chemistry. It is based on grafting functional monomers (usually hydrophilic) onto surfaces and can be carried out in three main steps as described below and shown in figure 4:

i) Polymers are first exposed to the plasma (typically argon, oxygen or helium) to activate the surface and produce radicals [72].

ii) The substrate is then kept under air atmosphere. Most of the formed radicals are oxidized leading to oxygen and peroxide groups used to initiate the grafting of the monomer [79,80].

iii) Afterwards, the substrate is dipped in the monomer solution under inert atmosphere and the solution is heated [80]. Oxygen in the monomer solution should be avoided because it can inhibit the reactions and heating is needed to enhance peroxide decomposition rate [12,77].

In some cases grafting can also be done in vapor phase. When the plasma treatment on polymers is completed, the gas flow is cutoff and monomer vapor is introduced into the chamber [81]. Since the plasma only produces radicals close to the surface of the polymers, plasma-grafting is restricted to the upper surface layers [12].

Surface grafting provides versatile techniques for introducing functional groups such as amine, imine, hydroxyl, carboxylic acid, and epoxide onto a broad range of conventional polymeric substrates, most of which
have a non-polar, less reactive surface. It is one method for decreasing the time-dependent effects of plasma treatment. The grafted monomers that are chemically bound to the surface are expected to prevent the hydrophobic recovery, reduce surface rearrangement, and increase treatment stability [22,82]. Most importantly, the functional groups introduced can be used for further reactions with small or large molecules through covalent or non-covalent linkage. It is, therefore, of interest to understand how plasma and grafting operating parameters affect the type and quantity of the desired functional group [77,78]. Acrylic acid (AA) has been most widely used in the plasma-induced grafting method for introducing carboxylic acid functions [22,77,81]. AA has a strong affinity for proteins leading to the formation of an inter-polymer complex. The interaction is robust, because of multivalent hydrogen bonding of carboxylic acids with peptide bonds and other proton-accepting and -donating side groups possessed by amino acids [81].

Figure 4: Schematic illustrating the concept of the plasma-induced grafting process.

5.3. Plasma polymerization/Plasma-enhanced chemical vapor deposition (PE-CVD)

Plasma polymerization is essentially a plasma-enhanced chemical vapor deposition process. It refers to the deposition of thin polymer films by vapor phase deposition through reactions of the plasma with an organic monomer gas (Fig. 5). The transformation of monomers into polymers encompasses plasma activation of monomers to radicals, recombination of the formed radicals and subsequent deposition and polymerization of the excited species on the surface of a substrate. Plasma polymers do not comprise repeating monomer units, but instead, complicated units containing cross-linked, fragmented, and rearranged units from the monomers. Consequently the materials formed by plasma polymerization possess unique chemical and physical properties different from that of conventional polymers [12]. The films can be formed on pretreated surfaces (plasma-activated) or on practically any substrate with good adhesion between the film and the substrate. The process gas can be either a pure monomer gas or a small amount of monomer mixed with a carrier gas (e.g. argon). Low surface energy polymer films can be prepared from fluorine-containing monomers, such as C₂F₄ and C₆F₆, and from silicon-containing compounds, such as SiC₆, Si₂C₃O, and Si₂C₂O [10,12]. High surface energy polymer films can be formed from oxygen-containing monomers, such as acrylic acid, acetone, methanol, formic acid, and allyl alcohol [10].

The advantages of plasma polymers include:
- They are chemically inert.
- The films are pinhole-free.
- Films can be formed on a variety of substrates including polymers, metal, glass, and ceramics.
- Films with uniform thicknesses varying from microns down to nanometers and multilayer films can be formed.
- They have very good mechanical and barrier properties against various gases and water vapor.
- The variety of organic substances that can serve as monomers makes plasma polymerization an extremely versatile tool for the deposition of polymeric thin films.
- Aging is not a big problem in plasma polymerized films because of their highly cross-linked nature that decreases the mobility of polymer chains.

However, different process parameters are needed to adjust the deposition rate and the properties of the obtained thin film. Its structure is highly complex and depends on many factors, including reactor design, position of the substrate in the plasma, flux and energy of the ion bombardment, power level, substrate temperature, frequency, monomer structure, monomer pressure, and monomer flow rate [10-12].

Figure 5: Schematic illustrating the concept of the plasma polymerization process.
5.4. General plasma applications in food packaging

Polymer materials are inexpensive, easy to process, and exhibit excellent bulk and mechanical properties. However, their chemical inertness and their low surface energy represent generally a great barrier for their food packaging applications [21]. Plasma treatment has been used to expand the applications and transform these inexpensive materials into highly valuable finished products by:

- increasing polar groups, wettability and surface energy,
- improving dyeability, sealability, printability and adhesion to other polymers or metals,
- enhancing peel strength,
- improving gas and water barrier properties,
- reducing swelling tendency,
- increasing mechanical resistance and shear strength,
- reducing migration of plasticizers, and
- increasing stability [7,10,21,72,73].

These particular advantages and properties of plasma-treated films are highly desirable in food packaging applications to minimize leakage, reduce the risk of microbial contamination, prolong the shelf-life of foods, and improve package integrity.

Plasma treatments can also be used to clean and disinfect food contact surfaces. They can kill bacteria and viruses and are inexpensive, fast, and relatively safe [12]. Compared to conventional sterilizing methods using autoclave, chemical solutions, and gases (e.g. ethylene oxide, hydrogen peroxide), plasma treatments are effective near room temperature and in a shorter time (several minutes) without damaging or leaving toxic residues on the materials [72,83]. They are subsequently recognized as one of the most promising alternatives, particularly for heat sensitive materials which need to be kept sterile after processing [7]. However, the effectiveness of plasma to inactivate microorganisms on inert surfaces will depend greatly on the equipment design and operating conditions like gas type, flow rate, and pressure [73].

5.5. Plasma applications in protein adsorption studies and in setting up antimicrobial surfaces

Plasma has found applications in the study of protein adsorption on surfaces and in setting up antimicrobial surfaces. However, such applications are few in the food sector as compared to the biomedical sectors. Therefore, examples will be presented in both sectors because of the similarities between the two disciplines. The plasma principle and process are the same for both applications and the interactions between the modified surfaces and the attached bioactive agents are also similar because the most used agents include proteins, peptides and enzymes. The only difference is the properties of bioactive compounds that change the targeted applications.

In adsorption studies, plasma treatments were used to evaluate the effect of surface characteristics on adsorption behavior. Sterrett and co-workers [84] studied the protein (albumin) adsorption on polyurethane elastomers modified by O2, CH4, CF4, and C2F6 plasmas. Plasma treatments using CH4 and/or C2F6 increased the contact angle for both substrates while those with O2 and O2/CF4 decreased the contact angle for the substrates considered. For substrates exhibiting smaller contact angles (hydrophilic surfaces), protein adsorption occurred to a greater extent. However, Kiae and co-workers [85] showed that C2F4 plasma-treated surfaces with low energy surfaces retained a larger fraction of adsorbed albumin than the higher energy surfaces. The low energy plasma-treated surfaces should have high interfacial energies in water, with correspondingly high driving forces for protein adsorption through hydrophobic interactions [85].

For setting up antimicrobial surfaces, plasma treatments were used to functionalize polyester and polyethylene terephthalate woven fabrics prior to peptide-activation [28;86]. They were also used to generate the functional groups needed to covalently immobilize the bioactive agents on surfaces [87-89].

Such technologies present therefore a great potential for the attachment of antimicrobials or bioactive compounds on surfaces. If applied to food packaging and processing surfaces, they could allow for increased shelf-life and/or safety of food products. The purpose would then be to select and optimize the proper plasma process for the desired type of surface modification.

6. Surface characterization methods

The type of analytical tools used in characterizing surface modified polymers depends on the anticipated nature of the modification, the specificity required, and the resources available. The study of surface interactions between peptides, materials and bacteria for setting up antimicrobial surfaces requires using techniques capable of investigating the quantity of adsorbed peptides on the surfaces, the antimicrobial activity of the peptides
before and after the adsorption on surfaces, the surface modification at each step of the process, and most importantly, the interactions between the peptides and the materials. The most commonly used characterization methods for such studies are discussed briefly.

6.1. Peptide assays
Several methods exist for the determination of protein/peptides concentration in solution, including Biuret reaction, Lowry method, Bradford assay, and Bicinchoninic acid (BCA) assay. They are all based on the change of color upon complexing with protein but the BCA assay is more sensitive and applicable than either Biuret or Lowry procedures [90,91]. It also has less variability than the Bradford assay. The BCA assay has many advantages over other protein determination techniques because it is easy to use, the color complex is stable, there is less susceptibility to detergents and it is applicable over a broad range of protein concentrations. In this method, Cu²⁺ ions are reduced to Cu⁺ ions by the peptide [90]. The amount of reduction is proportional to protein concentration. In alkaline environments, Cu⁺ ion combines with BCA to form a purple-blue complex with strong absorbance at 562 nm. In addition to protein determination in solution, the BCA assay is suited for determining surface bound or adsorbed protein [22]. However, some interfering substances like buffer additives, chelating agents, and solvents can affect the accuracy of the method and they should be taken into consideration in the development of appropriate standard curves. The assay has been frequently used to determine the amount of nisin adsorbed or released from surfaces such as the food packaging poly(butylene adipate-co-terephthalate) film [92], bone cement [93], active polyethylene/polyamide/polyethylene film [94], agarose gel [95,96], and antimicrobial multilayer films (ethylcellulose/hydroxypropylmethylcellulose/ethylcellulose) [51].

6.2. Antimicrobial activity
In order to verify the efficacy of antimicrobial treated films, the antimicrobial activity should be assessed for the antimicrobial agent alone, for the films before treatment, after each surface treatment, and after the functionalization with the bioactive compounds. Such controls are needed to ensure that the peptides will preserve their activity after adsorption and that the activity of antimicrobial films is due to the presence of the bacteriocin and not to a change in the polymer surface chemistry during the modification process.

The minimum inhibitory concentration (MIC) method is commonly used to check the activity of the antimicrobials alone such as peptides [97-99]. MIC can be determined by broth or agar dilution methods. The broth dilution method consists of seeding a microplate or a series of tubes containing growth medium with the target microorganism and with different concentrations of the antimicrobial. The microplates or tubes are incubated at a specific temperature and for a predetermined period of time. The optical density of the microplates is then measured with a microplate reader and the turbidity of the tubes is visually inspected for microbial growth. MIC is the lowest concentration of peptide resulting in the complete inhibition of growth of a test microorganism [98]. In the agar dilution method, the principle is the same but the tubes or microplates are replaced by agar plates and the MIC is determined as the lowest concentration of the agent resulting in a clear zone of inhibition [100].

To assess the antimicrobial activity of the films, agar diffusion assays and culturability loss tests have been the most widely used [3,23,58,63,101]. In the agar diffusion assay, the test film is placed on a solid agar medium inoculated with the test microorganism. The plate is then incubated and the inhibition of growth below or around the sample is used to determine the antimicrobial activity of the sample. The method is simple and effective but it gives only a qualitative assessment of the antimicrobial activity. Though, it can be considered quantitative if the diameter of the clear zones around the films is measured [3]. The size of the inhibition zone is proportional to the concentration of diffused agent from the film but it is affected by many factors, such as nisin-sensitive strains, amount of added agar and surfactant, growth rate of the indicator organism, diffusion of the antimicrobial agents, and pre-diffusion step [102]. The agar plate test method simulates the wrapping of foods and may suggest what can happen when films come into contact with contaminated surfaces and the antimicrobial agent migrates or diffuse from the film to the food. Therefore, this method is suitable for peptides capable of desorbing from surfaces but is not applicable for testing the activity of immobilized agents [23]. In the culturability loss tests, liquid media (buffer, growth media or foods) are inoculated with the target microorganisms and the antimicrobial film. The flasks are incubated with mild shaking and samples are taken over time and enumerated to measure the reduction of culturable cells [3,101]. However, the ratio of film surface area to volume (of product or media) must be considered in this test. From an antimicrobial standpoint, high surface/volume ratios should increase the activity of antimicrobial films. But in real packaging applications, it may be impractical because the packaging surface area is predefined upon the products’ size and...
volume requirements. Moreover, tests in buffer may be misleading since sensitive cells in nutrient-poor media may recover if nutrients are present as in growth media or foods.

Numerous studies concerning the antimicrobial activity of packaging materials have been reported and different methods of antimicrobial activity determination have been used. There is, however, no agreement upon standard methods to determine the effectiveness of antimicrobial food packaging. It is therefore difficult to compare the results of these studies because of substantial variations in the bioactive compounds, in the packaging matrix, in the test microorganisms, and in the test methods [3,23].

6.3. X-ray photoelectron spectroscopy (XPS)

XPS or electron spectroscopy for chemical analysis (ESCA) determines the atomic composition of the top few nanometers of the surface. It provides data in the ca. 1–10 nm surface layer [103]. The sample is bombarded by monochromatic X-ray photons and the binding energy of emitted photoelectrons is calculated. The resulting spectrum is a plot of intensity (arbitrary units) versus binding energy (eV). The binding energy can identify the element and its oxidation state. The intensity of the ejected photoelectrons relates directly to the material surface atomic distribution and can therefore be used to quantify the percent atomic concentration and the stoichiometric ratios [22,104]. In addition to determining and quantifying the surface atomic composition, this technique can be used to identify the presence of specific functional groups. The overall shape of XPS peak depends on the chemical environment of the element and is determined by the distribution and proportion of each existing functional group [104]. Those functional groups can also be used for the peak decomposition of mixed surfaces using curve-fitting models. For example, if a surface is composed of two polymers, such models allow the evaluation of the proportion of each polymer at the surface [105,106].

XPS has been extensively used to characterize and quantify the surface chemical composition of polymers and classical materials. Recent applications include biomaterials, biological, and bio-organic systems (peptides, proteins, microorganisms, polysaccharides, food) [61,52,107-110]. Therefore, XPS provides a promising tool in interactions studies for confirming and quantifying the surface chemical modification and the peptides adsorption.

6.4. Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS is a powerful technique for analyzing the chemical structure composition of the uppermost molecular or atomic layer of a solid surface. It analyzes the fragments emitted from the first 1–5 top monolayers [111]. It has unique features combining surface sensitivity, chemical specificity, and high spatial resolution [79,112,113].

In secondary ion mass spectrometry (SIMS), the sample surface is sputtered by an ion beam and the emitted secondary ions are analyzed by a mass spectrometer. Depending on the sputtering rate, we can differentiate between static and dynamic modes. In dynamic SIMS, a high-energy primary ion beam (> 10^13 ions/cm²) is applied for a relatively short time on the sample. This erodes away the material continuously from the surface to the bulk and allows a depth profiling of its chemical composition [79,104]. In static SIMS, the primary ion dose must not exceed 10^13 ions/cm² to maintain sensitivity to the uppermost monolayers, to minimize sample damage and to promote the emission of large organic fragments [113]. ToF-SIMS is the main experimental variant of static SIMS that emerged as a technique of potential importance in surface science. For ToF-SIMS, a pulsed primary ion beam is used. The time of flight of the emitted secondary ions allows their separation in a mass spectrometer according to their mass to charge ratio. The resulting spectrum depicts signal intensity versus mass to charge ratio (m/z) and can be used to measure relative intensities of the chemical species.

ToF-SIMS can be used for surface chemical identification, imaging mode and semi-quantitative analysis.

- Surface chemical identification

The mass to charge ratio (m/z) of the species acquired in a ToF-SIMS experiment, yield positive and negative secondary ion mass spectra. By evaluating the masses of the signals, peaks can be often identified from the molecular ion of the analyte, fragments of the molecular ion, polymer repeat units, end groups and ions of any other components that may be in the samples [112,113]. Such characteristic peaks are used for the identification of the molecular structure and chemical composition of the surface.

- Imaging mode

Static ToF-SIMS can be done in imaging mode. The chemical composition of a sample can be mapped and a full mass spectrum is collected at each pixel in the image. After data acquisition, a specific secondary ion or a combination of ions can be selected and their surface distribution mapped. In addition, a region of interest from the total ion image can be identified and the mass spectra from the pixels in that region can be summed.
allowing spectral evaluation with restored sensitivity and dynamic range [113]. Such chemical maps can be also used for assessing surface homogeneity [22].

- Semi-quantitative analysis

ToF-SIMS is not a direct quantitative method due to the influence of the matrix effects on ion yields and the preferential ionization of one species over another. The intensity of a given fragment depends on its surrounding environment and is not necessarily directly proportional to its concentration on the surface. However, for most organic surfaces such as polymeric biomaterials and adsorbed protein films, these matrix effects are minimal [113]. Moreover, such analysis can be done by determining the relative amount of components at a surface of a sample and by rationing representative ions, elemental or molecular species [113,114].

However, ToF-SIMS data handling is complex and presents substantial challenges with interpretation. Imaging mode generates an enormous amount of data and a typical mass spectrum contains a huge number of peaks. Yet it is within this complexity that information about sample composition, molecular orientation, chemical bonding, and sample purity is contained. The challenge is how to extract this information from the spectra and images. In order to address and reduce such complexity, the multivariate analysis can be applied to process ToF-SIMS data. Examples of these methodologies include: principal component analysis, partial least squares, multivariate curve resolution, maximum auto-correlation factors, neural networks, latent profile analysis, mixture models, and discriminant analysis [113,115,116]. Another approach for ToF-SIMS data analysis is multi-method combination. Together static ToF-SIMS and XPS provide a powerful complementary approach to biomaterial surface analysis and represent the two most widely used surface analysis techniques [113]. ToF-SIMS can be used to complement XPS results by offering identification of chemical species and it may also be used to differentiate samples that have similar XPS spectra [22]. ToF-SIMS is more surface sensitive than XPS and characterizes the uppermost layers of the surface at lower sampling depth.

This technique has found many applications in the study of protein and peptide adsorption on surfaces [61,117-121].

6.5. Fourier-transform infrared (FTIR) spectroscopy

FTIR spectroscopy uses infrared radiation (IR) to determine the chemical functionalities present in a sample. When an infrared beam hits a sample, chemical bonds stretch, contract, and bend causing it to absorb IR radiation at specific wavenumber. The resulting plot is of absorbance (or transmittance) versus wavenumber. In this way, infrared spectra show absorption peaks that are characteristic of particular molecules and the way they are bonded to the surface. The advantages of this technique are that it does not require ultra-high vacuum conditions, as do XPS and ToF-SIMS, and an analysis can therefore be conducted in less than ten minutes. However, FTIR utility is limited by the micrometer range probed by the method, which is often too deep to detect modification or adsorbed agents at the uppermost layers of the surface. Modified surface layers with thickness from only several to tens of nanometers, cannot be observed by FTIR with sampling depth ranging from several hundred nanometers to more than 1 μm [79,112]. In such cases, other techniques like XPS and TOF-SIMS with much smaller sampling depths are needed. Consequently, this technique may not be relevant for the study of surface interactions and peptide adsorption but it can be used to monitor migration of functional groups to the polymer bulk, to determine the depth of surface modification, and to confirm functionalization of plasma-treated surfaces [10,122].

6.6. Dye assays

In a complementary approach to other techniques (FTIR, XPS, SIMS, etc.), dye assays or colorimetric methods are used to measure the amount of functional groups on a surface. Two frequently used methods for determining the surface density of carboxyl and amino groups on surfaces are respectively, toluidine blue O (TBO) and methyl orange (MO) dye tests. They are based on ion exchange mechanism and on electrostatic interaction between the functional group target and the dye. TBO is a positively charged molecule that can combine with a carboxyl group in alkaline solution to form a stable electrostatic complex. The complexed dye TBO molecules can be detached from the surface by dissolving in acetic acid or other organic solvents [77,79]. Similarly, MO is a negatively charged dye and can combine with positively charged amino groups on the material’s surface under acidic conditions [79,123]. The combined MO molecules can be desorbed in potassium carbonate solution or other organic solvents. The amount of TBO and MO can be determined by measuring the optical density and using a standard calibration curve. The amount of functional groups is calculated by assuming a combination ratio of 1:1 between TBO and –COOH and between MO and –NH₂. However, these methods remain less sensitive than XPS and SIMS and the pH control is critical to minimize error. TBO has been mostly used to determine the surface density of carboxylic acid functions on acrylic acid grafted and oxygen plasma-treated
surfaces [77,78]. MO dye assay was used to measure the amino groups’ density on ammonium plasma-treated surfaces [123].

6.7. Contact angle and surface energy measurements
Contact angle measurements are surface sensitive, providing information about the outermost few ångströms (= 5 Å) of the sample surface [78,81]. The contact angle measurement can be static or dynamic. The former is a measurement where the liquid is not in motion at the solid/liquid interface. The latter is a measurement where the liquid front is in motion with respect to the solid surface [81]. Most applications and especially in the scope of this review include the static measurement.

Water contact angle determines surface hydrophilicity/hydrophobicity by measuring how much a droplet of water spreads on a surface. The lower the contact angle, the more hydrophilic the surface is. When a surface has more polar groups introduced to it, hydrogen bonding with the water becomes easier and the droplet spreads along the hydrophilic surface, resulting in a lower contact angle. While contact angle is a simple and rapid method, it is limited by its inability to distinguish between different hydrophilic functional groups and by the measurement errors including difference in operator measurement, inconsistent water pH and hardness, and changes in environmental temperature and humidity.

When the contact angle is measured using two or three test liquids including polar (water) and non-polar (diiodomethane) liquids, the surface energy can be determined. The solid surface energy is the sum of polar and non-polar (dispersive) contributions. It permits to take into account the effect of these two contributions on the surface properties and interaction processes [71,124,125].

Both contact angles and surface energy measurements have been widely used to confirm the introduction of polar groups into surfaces, to determine the hydrophilic/hydrophobic character of plasma-treated surfaces and to show the effect of plasma treatment type and conditions [11,71,78,124-127].

6.8. Zeta potential
Zeta potential is the most important technique used to acquire information on surface charge. When a charged solid surface is in contact with a liquid phase, an electrical potential develops at the interface. A double layer is established and divided into:
- the immobile (fixed) layer made of surface bound ionizable groups and tightly bound liquid phase ions of opposite charge, and
- the mobile (diffuse) layer made of loosely bound liquid phase ions of opposite charge.

A shear plane separates the layers from each other and the change in potential across this double layer is known as the electrokinetic or zeta potential [22,128] (Fig. 6). Commercial zeta potential or electrokinetic analyzers are available and bench top units can be set up as well.

**Figure 6:** Schematic of zeta potential showing the double layer formation at the solid/liquid interface.
The accumulation of surface charges (formation of the double layer) and the magnitude and sign of the zeta potential depend on:
- the type and the amount of dissociable functional groups on the solid surface,
- the pH of the electrolyte solution, and
- the interaction between the solid surface and the electrolyte solution.

Therefore, zeta potential measurements provide information not only about the surface charge but also about its chemical composition and acid-base character. However, they cannot be used to quantify the number or type of functional groups present and must be used in conjunction with other analytical tools to adequately describe changes in polymer surface chemistry. Zeta potential has found applications in confirming the introduction of cationic and anionic groups into plasma-treated or grafted surfaces [75,81,129] and in determining the charge and isoelectric points of surfaces [128,130]. Furthermore, it can be used to predict protein adsorption behaviors. The adsorbed mass can theoretically increase with increasing charge contrast between the surface and protein. Consequently, the surface charge would play an important role when electrostatic interactions are driving the adsorption phenomenon.

6.9. Atomic force microscopy (AFM)

AFM is used mainly for measuring surface topography and roughness. It is a very high resolution scanning probe method. An ultra-sharp AFM probe (tip) attached to a flexible cantilever, scans over the sample surface with sub-nanometer precision. Features on the sample surface, induce the cantilever deflection in the vertical and lateral directions as the sample moves under the tip. The AFM detects and records interactions between the tip and the surface. A surface topographical map is thereby generated and can be used for surface roughness calculation [22,131,132]. AFM can additionally provide high resolution three-dimensional images of solid surfaces and can work under different conditions such as ambient air, various gases, liquid, vacuum, low and high temperatures [81,112,132]. It has different modes including contact, tapping, and resonant or lateral modes, which can provide a deeper knowledge for different kinds of polymer surfaces [81,131]. The tapping mode (interrupted contact mode) is the most frequently used and is adapted for weakly adsorbed molecules and for soft surfaces because it minimizes the effects of friction and other lateral forces between the tip and the sample. In this mode, the cantilever/tip assembly is sinusoidally vibrated by a piezo-mounted above it, and the oscillating tip slightly taps the surface [131,132].

Disadvantages of AFM include limitations on the image maximum scanning area (around 150 x 150 µm). Another inconvenience is that at high resolution, the quality of the image is limited by the radius of curvature of the probe tip, and an incorrect choice of tip for the required resolution can lead to image artifacts [112]. Also, ultra-flat or rigid samples are desirable to obtain high atomic resolution [131].

AFM has been used to determine surface topography and roughness of plasma-treated surfaces [127,133]. It has also been used to study the surface topography of plastic films activated with bacteriocins [54]. In addition to evaluating surface topography, AFM can be used to investigate the interaction between the probe tip and the polymer-adsorbed or -grafted surface in an aqueous solution. Such applications permitted to measure the interaction forces between proteins and graft-polymerized surfaces [69].

In the scope of this review, AFM can be used to study the topography changes of surfaces after surface treatment and after peptide adsorption. Because of its high nanometer resolution in describing surface topography, AFM may help to explain the adsorption mechanisms of different antimicrobials on surfaces. It can also measure the force of interactions between the peptides and the surface.

6.10. Scanning electron microscopy (SEM)

SEM allows also the study of surface topography. When a sample is bombarded with electrons, it emits secondary electrons and X-rays. The intensity of the secondary electrons is detected to generate a high resolution three dimensional surface image. X-rays can be detected to conduct elemental analysis. SEM is not as surface sensitive as other techniques, and non-conducting polymers must be sputter-coated prior to analysis [22]. Nevertheless, it has often been used to measure surface topography of plasma-treated surfaces [71, 124,127,133,134].

6.11. Ellipsometry

Ellipsometry enables to study the kinetics of protein adsorption on surfaces and to characterize the thickness and structure of the adsorbed layer [79,112]. This is an optical technique that measures the changes in polarization state of a reflected light from its incident light. When a monochromatic linear polarized light is reflected by a
smooth surface, the polarization state will be changed, and will be further changed if a protein layer exists on the surface. The polarization state of the reflected light is related to many parameters including the protein layer thickness, which can be calculated through fitting the changes of polarization with a mathematical model. However, a great limitation of ellipsometry is that it cannot distinguish between polymers and proteins that have similar refractive indices. Only very smooth surfaces with strong reflection ability and different refractive index from that of proteins such as silicon or silicon dioxide can be used. Thus, the technique is not practical for the analysis of polymer surface materials [112,135] and has been used to study nisin adsorption on silicon surfaces [62,68].

7. Further considerations
Setting up active food packaging by adsorbing peptides on plasma-treated surfaces will need to take into consideration further challenges related to the peptides, plasma surface treatment, appropriate testing methods, applications in food, regulations, and cost.

7.1. Peptides
Peptides should be able to preserve their antimicrobial activity after adsorption on the surface and they should not change the film’s performance or mechanical, barrier, and optical properties [3,17]. Their usage should be optimized or combined with other control factors to increase their effectiveness and to avoid bacteria cell resistance. The greatest restriction to the development and application of natural antimicrobial agents is cost.

7.2. Plasma surface treatment
The challenges encountered in plasma treatment can be summarized as follows:
- Most plasma treatments are performed under low-pressure and therefore require vacuum systems. This increases capital and operational costs of the plasma equipment and presents complications for continuous processes and for scaling up from laboratory size to a large scale industrial setting [22].
- The process parameters are highly system-dependent; the optimal parameters developed for one system usually cannot be adopted for another system. Consequently, it is extremely difficult to repeat and compare results between laboratories and from a large variety of plasma systems [10,22].
- It is very difficult to control precisely the amount of a particular functional group formed on a surface [10]. Due to the multitude of elementary reactions occurring simultaneously in the plasma process, it is complicated to calculate in detail the plasma’s physical and chemical behavior and especially its interactions with the surface.

Overcoming some drawbacks of plasma systems will increase their potential for surface treatment applications. In order to avoid vacuum conditions, the use of atmospheric pressure plasmas was considered as an alternative [24]. Atmospheric pressure plasmas can operate in a wide range of temperature and pressure. However, they can be controlled to operate like low-pressure cold plasmas without generating extensive heat in their surroundings [83]. As a result, they can also be suitable for the processing of organic compounds and for surface modification of polymer materials.

A solution for problems related to complex plasma processes requires a good knowledge of reactor hardware and design criteria [136], as well as future progress in simulation techniques and creation of sophisticated mathematical models able to predict real plasma processes.

7.3. Appropriate testing methods
There are many advanced techniques that have potential applications for surface analysis but there are still many unanswered questions in this field. The challenges rely on the choice of the adapted technique for the corresponding problematic, the proper combination of complementary techniques, as well as being able to process the complex data provided by those techniques. In addition, analyzing results from different combined techniques requires a thorough understanding of each method’s principle, advantages, and limitations. For example FTIR, XPS, and ToF-SIMS have very different sampling depths and sensitivities that should be considered for interpreting the results correctly.

7.4. Applications in food
Testing the microbial growth in synthetic media should be followed by growth in the targeted foods. The food components can alter the antimicrobial activity of the bioactive agents or inhibit their release from the packaging [2,3,17]. The storage of food under different temperatures and environmental conditions can also
affect the activity of the antimicrobial films and proper simulation tests need to be carried out [1,17]. In addition, it is crucial to study the kinetics of release of the bioactive substances in order to understand or to modulate film activity and to investigate which type of food could be protected efficiently using these systems of active films.

7.5. Regulations
Regulations related to active packaging differ from country to country and are detailed in previous reports dedicated to this topic [137,138]. While active packaging is not subject to any special regulatory concern in the United States, the regulation of such packaging material in Europe is still evolving. In the US, antimicrobials in food packaging that may migrate to food are considered food additives and must meet the food additive standards [3,7]. Unlike the US, EU countries regulate substances added to or used in packaging separately from food additives [7]. To be on the European market, such systems should comply with specific regulations concerning the active packaging [2,137,138]. Consequently the regulatory issues should be addressed too because they present complications limiting the commercial availability of antimicrobial packaging.

7.6. Cost
Even though there is no published data on the cost of antimicrobial films, they can be expected to be expensive due to the cost of plasma technological investment and the usage of natural antimicrobial peptides. This may restrict options for commercialization, especially for small and medium sized businesses. However, such costs are negligible compared to the economic loss associated with foodborne illness outbreaks. Moreover, these cost increases are counterbalanced by benefits such as:
- Reductions in waste due to the improved quality and shelf-life of products.
- Lower waste disposal expenses of chemicals generated by wet chemistry processes.
- Environmental considerations associated with such environmentally benign processes.
- Replacement or simplification of aseptic packaging process and chemical sterilization of the packages. Packaging materials will have self-sterilizing abilities due to their own antimicrobial effectiveness.
- Improved additional functional properties of plasma-treated food packaging films (See section”5.4.”).

Therefore, a comprehensive assessment of specific costs and benefits is an essential next step in establishing the commercial application of innovative packaging systems. Recognition of the benefits of active packaging technologies by the food industry and increased consumer satisfaction will open new frontiers for active packaging technology. As interest in the field grows; so will availability and cost effectiveness of antimicrobial packaging.

Conclusions
Antimicrobial surfaces and packaging are gaining interest from researchers and industry due to their potential to provide quality and safety benefits. In this review, we summarized the methodology, advantages, disadvantages, testing methods, applications, and considerations related to antimicrobial food packaging systems in general, and specifically to peptide adsorption on plasma-treated surfaces. Obviously, there is much work to be done. The methods for setting up antimicrobial packaging are still in the developmental stages and the peptide/protein adsorption on surfaces remain a complicated unclear phenomenon that need to be studied further. In order to advance in this field, investigating the complex interactions at the interfaces is a prerequisite for later successful packaging applications. This implies the need for continued research in setting up new functionalized surfaces, better modulating surface treatment processes, and developing surface characterization methods. More importantly, the effective participation and collaboration of research institutions (materials scientist, plasma physicists, chemists, biologists, bioengineers…), industry, and government regulatory agencies are imperative.

References

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